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■ BIOTECHNOLOGY RESOURCES

CCR Molecular Modeling Core

The CCR Molecular Modeling Core (MMC), a free-of-charge, centralized scientific resource for the intramural research community, serves as a clearinghouse for molecular modeling knowledge and available tools. The MMC informs CCR scientists how modeling and related computational methods could benefit their work, assuring that appropriate methods and strategies are adopted. The MMC facilitates collaborations with other computational scientists with the requisite skills or, when appropriate, performs the studies itself. Affiliated core staff members are particularly experienced in the following: protein homology modeling, evolutionary multi-family sequence alignment and statistical analysis, molecular docking and virtual drug screening, molecular dynamics simulation, ion channel and membrane protein modeling, and DNA structure and protein interaction modeling.

The Role of Modeling

Basic science research and translational medical opportunities are increasingly being driven by discoveries at the biomolecular level. Revolutionary advances in genome sequencing and system biology are uncovering new genes and interactions between their protein products, often presenting novel protein targets for drug development. Knowledge of a molecule's structure is often key to understanding its function. The three-dimensional structure of proteins and nucleic acids provides a physical framework to integrate disparate experimental results. Although structure

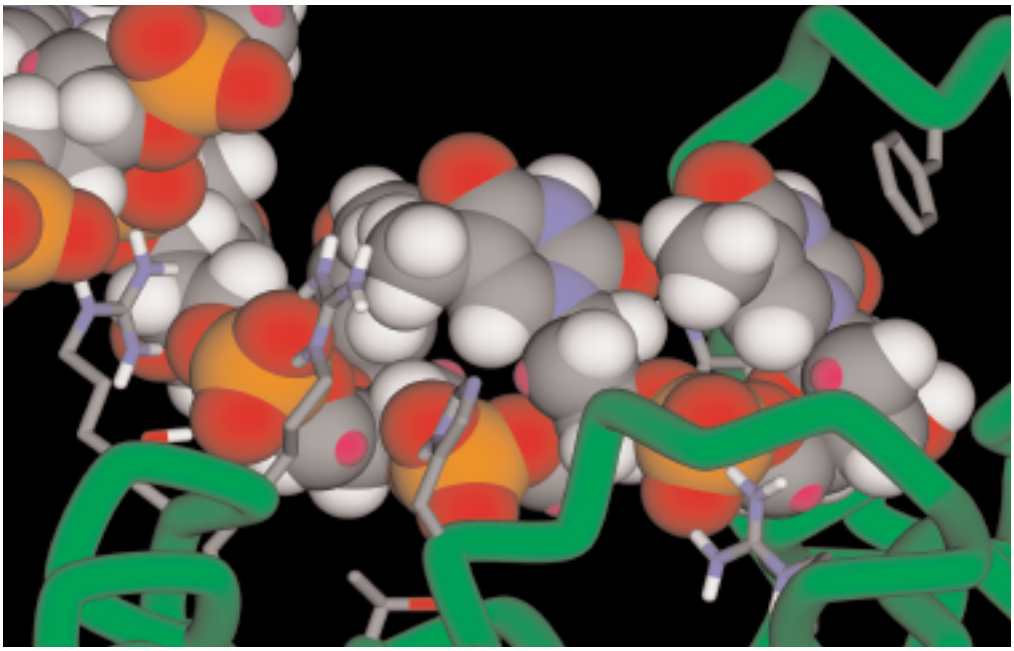

Figure 1. Model of Bloom syndrome–associated helicase bound to single-stranded DNA. The DNA is shown as space-filling atomic spheres. The protein backbone is shown as a green tube. The model suggests several amino acid sidechains (shown as sticks) that can be mutated to test their effects on enzyme activity.

determination by the experimental methods of X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy remains the gold standard for obtaining accurate structural information, these methods are still technically demanding. Fortunately, structural genomics programs (with the goal of increasing the number of known protein folds) are adding to the already large database of known protein structures. Consequently, computational modeling methods, such as protein homology modeling, together with powerful bioinformatics tools, are increasingly likely to aid in predicting the structure of a protein of interest. In addition, whereas, crystallography and NMR typically yield rather static snapshots of a protein structure, computational modeling tools can predict functional conformational changes, identify active sites, and predict drug binding specificities. Modeling has proven to be of greatest benefit when conducted hand-in-hand with experimental studies: Models developed from experimental data serve to stimulate new ideas about how the molecule functions, and help to direct the design of new experiments to test these hypotheses. For example, site-directed mutagenesis studies and analysis can be accomplished far more efficiently and convincingly with a model of the structure in hand.

Recent Projects

One current project of the MMC concerns modeling of human helicases in collaboration with Curtis Harris, MD, of the Laboratory of Human Carcinogenesis. Helicases form a superfamily that couples ATP hydrolysis to the unwinding of duplex DNA and RNA. Since these processes are essential for protein expression and cell replication, it should be no surprise that naturally occurring mutations in some human helicases have been linked to increased rates of cancer. The short-term goal of this project is to understand the mechanisms by which helicases interact with oligonucleotides, and how other proteins such as p53 (a major regulator of cell growth) modulate their functions. Unfortunately, the structures of only a handful of helicases have been experimentally determined, and to date, all of these are from microorganisms (archaea, bacteria, yeast, and viruses). Furthermore, the crystal structure that provides the most information on interaction with DNA is from a relatively distant subfamily. Thus, a multi-step modeling approach was undertaken whereby the amino acid sequences of the human helicases were first modeled on the known structures of the RecQ-like proteins. Subsequently, the structural domains were superimposed on the superfamily I helicase structure according to similarities in the three-dimensional fold. As shown in Figure 1, this provided a model showing how human helicases can bind to partially unwound DNA and which residues may interact directly with DNA. Experiments to measure these effects are now being conducted as a test of the model. Additional comparisons of models of the hexameric structure of the helicases with hexametric SV40 large T antigen have suggested which residues may be involved in binding to p53. A long-term goal of this project is to develop drug-like compounds that are able to modulate the activities of both mutant and wild-type helicases as a treatment for cancer and associated diseases.

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■ MOLECULAR BIOLOGY

How Our Thumbs Become Different from Our Pinkies

Chen Y, Knezevic V, Ervin V, Hutson R, Ward Y, and Mackem S. Direct interaction with Hoxd proteins reverses Gli3-repressor function to promote digit formation downstream of Shh. *Development* 131: 2339–47, 2004.

Developmental signals and regulators are not unique to the developmental stages of an organism. Although they are first employed during embryogenesis, they are later used repeatedly in both normal and pathologic processes, such as tissue renewal or regeneration and oncogenic transformation. For example, deregulation of the Hedgehog signaling pathway in stem or early progenitor cells, mediated by Gli family transcription factors, leads to sporadic cancer in several organs (e.g., CNS, stomach, pancreas, prostate, skin), as well as genetic diseases such as Gorlin's syndrome, in which skeletal abnormalities accompany a neoplastic diathesis. The frequent abnormal activation of Gli-regulated transcription programs during oncogenesis makes these factors prime targets for cancer therapeutic intervention.

Our interest in understanding the normal developmental function of the Hedgehog signaling pathway has led us to examine its influence on digit identity. Different digits arise from initially similar condensations that later segment, grow, and differentiate to acquire a unique identity. Both digit number and pattern are regulated by posteriorly secreted Sonic Hedgehog (Shh) protein acting as a dose-dependent morphogen: the highest levels of Shh specify the most posterior digit (pinky), whereas the lowest levels specify the most anterior digit (thumb). How is this quantitative gradient interpreted by cells to direct the formation of specific digit types?

Shh prevents the cleavage of full-length (FL) Gli3 activator; cleavage of FL Gli3 transforms it into a truncated form (TR)

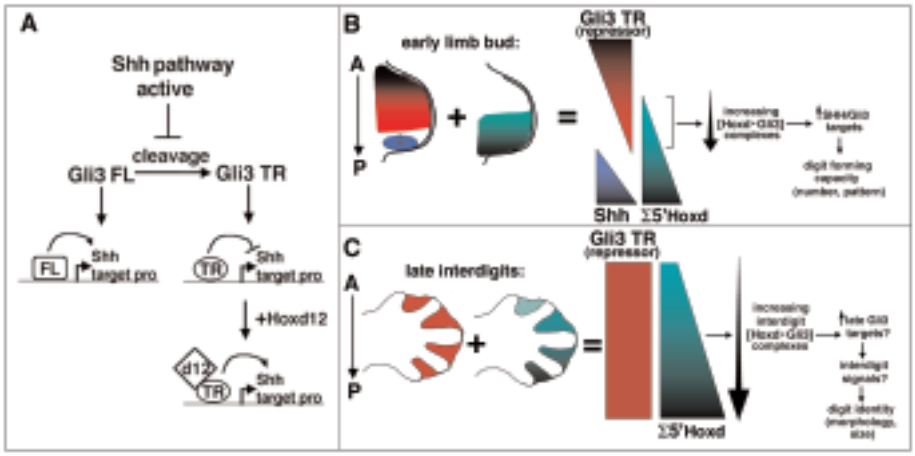

Figure 1. Model for role of Gli3-Hoxd interaction in digit formation. (*A*) Effect of Hoxd interaction on Gli3-regulated transcription. Hoxd12 (d12) converts Gli3 truncated form (TR) from a repressor to an activator of its target promoters (pro) through protein interaction and without any need for Hoxd12 binding to promoter (pro)DNA. (*B* and *C*) Overlap of total 5′Hoxd proteins (Σ5′Hoxd) with Gli3 repressor in the early limb bud (*B*) and interdigits (*C*) and possible effects on regulation of digit number and morphogenesis (identity). A, anterior; P, posterior; FL, full-length Gli3; Shh, Sonic Hedgehog protein.

that represses Shh target genes. Analysis of *Shh*$^{-/-}$/*Gli3*$^{-/-}$ mutants has revealed that antagonizing Gli3 TR (repressor) accounts for much of Shh function in digit formation, and in fact, the loss of digits in *Shh*-null embryos can be rescued by completely eliminating *Gli3* (Litingtung Y et al. *Nature* 418: 979–83, 2002; te Welscher P et al. *Science* 298: 827–30, 2002). However, digits formed in the absence of Gli3 lack distinct identities and are all similar and dysmorphic regardless of whether Shh is present. Clearly, some critical determinant of digit identity is missing. It is presumed that this determinant is FL Gli3 (activator), although the evidence for this is controversial.

5′*Hoxd* genes are expressed in overlapping posterior zones in the early limb bud and regulate digit pattern downstream of Shh. Despite their nested expression, there is no combinatorial *Hox* "code" for digit identity. When null alleles are analyzed, 5′*Hoxd* genes behave semi-redundantly and have incremental, additive effects on digit morphology. In contrast, enforced *Hoxd* gene expression increases digit number, often with changes to posterior identity. Either elevation of *Hoxd* levels or elimination of Gli3 leads to activation of the Shh pathway, but in the former case, digit identity is preserved, whereas in the latter it is lost.

To dissect the regulatory hierarchy between Shh, Gli3, and 5′*Hoxd* genes, we evaluated the effects of transgenic *Hoxd12* misexpression in the context of varying *Gli3* in *Gli3*$^{+/+}$, *Gli3*$^{+/-}$, and *Gli3*$^{-/-}$ mouse embryos. Altering the balance between *Gli3* and *Hoxd12* had strongly synergistic effects on digit formation that required the presence of some functional Gli3 protein (albeit at reduced levels); transgenic *Hoxd12* had no effect on digit phenotypes in *Gli3*$^{-/-}$ embryos. Some effects of transgenic *Hoxd12* were greatly exacerbated by reduced *Gli3* but lost in the total absence of *Gli3*, which is most readily explained by a physical interaction. We confirmed this by co-immunoprecipitation of endogenous Hoxd12 and Gli3 proteins from limb bud lysates. Mapping interaction domains showed that the homeodomain of only certain Hox protein subclasses (5′, posterior) interacted efficiently with Gli3, but the DNA binding function of the homeodomain was completely dispensible. The effect of interaction on Gli3-regulated transcription was evaluated in co-transfection assays. Hoxd12 converted Gli3 TR (repressor) into an activator of Gli-responsive promoters in a stoichiometric, dose-dependent fashion (Figure 1, part A), independent of Hoxd12-DNA binding. Several 5′Hoxd proteins interact with Gli3, suggesting that the sum of these interactions sets the expression level of Shh/Gli3 target genes (along with FL Gli3-activator). Where free Gli3 TR (repressor) is in excess, repression occurs; where Hoxd-Gli3 interactions prevail, dose-dependent activation occurs.

When do Gli3-Hoxd proteins interact in the limb? Gli3 predominates anteriorly and overlaps with Hoxd expression in the mid-region of the early limb bud; their interaction may serve to extend Shh effects anteriorly (Figure 1, part B) and increase digit number. Later, 5′*Hoxd* genes are expressed differentially across the interdigits along with high, uniform levels of Gli3 TR (repressor) in all interdigits (Figure 1, part C). Notably, differing interdigit signals actively regulate the identity of forming digit condensations at these later stages (Dahn RD et al. *Science* 289: 438–41, 2000), and these secreted signals may be a late target of Gli3:Hoxd interactions.

Our results support a stoichiometric model in which varying [Gli3]:[total Hoxd] protein ratios in different parts of the limb bud lead to differential expression of Shh/Gli3 target genes by altering the balance between "effective" Gli3 activating and repressing functions. This model provides a mechanistic basis for the quantitative, dose-dependent nature of *Hoxd* gene function in regulating digit pattern and may have relevance for human genetic diseases due to *Gli3* mutations. Pallister-Hall Syndrome and Post-Axial Polydactyly arise from mutations producing a truncated, constitutive Gli3 repressor. Yet, Shh function is not blocked in the limb in these syndromes, which instead show polydactyly. Interaction with 5′*Hoxd* genes in the limb may thwart a *Gli3* mutation that behaves as a dominant repressor elsewhere. Our results also raise the possibility of other Gli-Hox family member interactions, which may modify the activity of the Shh pathway in specific contexts.

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Lineage Differentiation in the Thymus: The Short Road and the Long Road

Liu X and Bosselut R. Duration of TCR signaling controls CD4-CD8 lineage differentiation *in vivo*. *Nat Immunol* 5: 280–8, 2004.

T lymphocytes, which play an essential role in immune response, hold much promise in our attempts to use the immune system to fight cancer. These cells recognize peptide antigens through a specific T-cell receptor (TCR) and generally belong to either of two lineages: CD4 T cells (which provide help to other components of the immune system such as B cells or macrophages) and CD8 T cells (which are cytotoxic and able to directly kill cells expressing their target antigen).

Because CD4 and CD8 T-cell lineages develop in the thymus from precursors that express both CD4 and CD8 molecules, the question arises as to how immature $CD4^+CD8^+$ thymocytes choose either the CD4 or the CD8 lineage. Experiments performed in the laboratories of Alfred Singer, MD (NCI), and Ronald Germain, MD, PhD (NIAID), have suggested that this lineage decision is determined by the kinetics or duration of TCR signaling; that is, persistent signals would promote CD4 choice and transient signals CD8 choice (Brugnera E et al. *Immunity* 13: 59–71, 2000; Yasutomo K et al. *Nature* 404: 506–10, 2000).

To test this hypothesis directly *in vivo*, we manipulated the duration of TCR signaling during intrathymic T-cell development. Our approach was to genetically restrict the developmental window of expression of Zap70, a tyrosine kinase required for TCR signaling. By expressing Zap70 from transgenic regulatory elements that are active only in $CD4^+CD8^+$ thymocytes, we limited the duration (understood as the developmental window) of TCR signaling to these cells. These limited signals allowed thymocytes to initiate their differentiation but not to complete it, causing a developmental block before the generation of mature CD4 or CD8 T-cell populations (Liu X et al. *J Exp Med* 197: 363–73, 2003).

We then examined if and how these arrested cells underwent lineage choice (Liu X et al. *Nat Immunol* 5: 280–8, 2004). We found that all arrested thymocytes had chosen the CD8 lineage; in fact, single-cell analyses showed that even cells that had downregulated CD8 gene expression and therefore appeared as CD4 "wannabes" expressed the CD8 marker perforin, unlike their counterparts in wild-type mice. Furthermore, we demonstrated that TCR signals confined to the $CD4^+CD8^+$ stage promote CD8 lineage choice even when induced by intrathymic TCR ligands that normally promote CD4 lineage choice (that is, self-peptides complexed to class II major histocompatibility complex molecules).

These data demonstrate that developing T cells use the duration of TCR-generated signals as a criterion in their lineage-choice decision. What does duration of signaling mean at a mechanistic level? There are two schematic possibilities. The first is that during thymocyte differentiation, distinct sets of decisions take place sequentially in response to the same TCR signaling gradient. Early TCR signals would cause $CD4^+CD8^+$ thymocytes to initiate their differentiation and to reach a developmental stage where they have become competent for lineage choice. At this stage, subsequent TCR signals would be translated into a lineage decision. In this perspective, thymocytes do not really sense the kinetics of TCR signals: even though TCR signals need to persist longer for CD4 lineage choice than for CD8 lineage choice, the only gradient that thymocytes sense is one of TCR signal intensities.

The other, more speculative possibility is that thymocytes sense signal kinetics independently of signal intensity. A precedent for such a kinetic sensor is the ability of cells to discriminate between persistent and intermittent transmembrane calcium fluxes through distinct kinetic requirements for the calcium-dependent activation of two transcription factors, NF-κB and NFAT. Although there is little evidence that these factors participate in lineage choice, it is conceivable that other intracellular signaling intermediates could be sensitive to kinetic changes in TCR signals. The challenge now is to identify intracellular targets of TCR signals to find such putative kinetic sensors.

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■ IMPORTANT NOTICE

E-Distribution

All staff members will start receiving *CCR Frontiers in Science* electronically via email beginning in March 2005.

17-AAG Potentiates *In Vitro* and *In Vivo* Radiation Cytotoxic Sensitivity in Cervical Tumor Cells

Bisht KS, Bradbury CM, Mattson D, Kaushal A, Sowers A, Markovina S, Ortiz KL, Sieck LK, Isaacs JS, Brechbiel MW, Mitchell JB, Neckers LM, and Gius D. Geldanamycin and 17-allylamino-17-demethoxygeldanamycin potentiate the *in vitro* and *in vivo* radiation response of cervical tumor cells via the heat shock protein 90-mediated intracellular signaling and cytotoxicity. *Cancer Res* 63: 8984–95, 2003.

Tumors exhibit considerable genetic plasticity and can quickly adapt to the cytotoxicity of many anticancer agents. Many of the signaling pathways and oncogenes upregulated or altered during cellular transformation confer this plasticity and resistance to environmental insult. Therefore, the development of new anticancer agents involves identifying novel molecular targets to improve therapeutic outcome, ideally with efficacy both alone and when combined with existing agents that have multiple targets.

The heat shock protein 90 (HSP90) has emerged as a molecular target candidate due to its role in stabilizing protein complexes involved in signal transduction pathways related to proliferation, cell cycle progression, and apoptosis, and processes characteristic of the malignant phenotype, including invasion, angiogenesis, and metastasis. Ansamycin antibiotics inhibit HSP90 activity, causing selective degradation of several intracellular proteins regulating these physiological functions and malignant processes. Because HSP90 has been previously suggested as a molecular target for ionizing radiation (IR), it seemed logical to hypothesize that the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) may enhance tumor cell susceptibility to the cytotoxicity of IR.

To address this hypothesis, we initiated a series of translational/pre-clinical

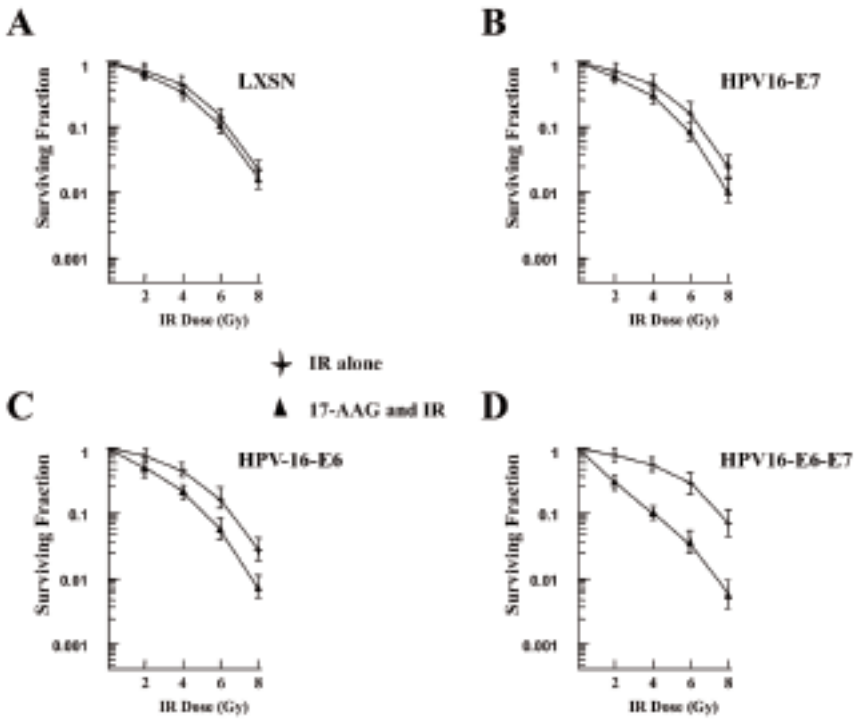

Figure 1. 17-allylamino-17-demethoxygeldanamycin (17-AAG) is preferentially cytotoxic in transformed cells. Clonogenic cell survival assays depicting effects of 12-hour pretreatment with 17-AAG on radiation response of LXSN (empty vector) (*A*), or viral expression vectors encoding HPV16-E7 (*B*), HPV16-E6 (*C*), or both HPV16-E6 and HPV16-E7 (*D*). Although not a perfect model system to address our research question, these four cell lines resemble non-transformed or benign cells (LXSN and HPV16-E7), malignant (HPV16-E6), and frankly malignant cells (HPV16-E6/HPV16-E7) and provide a cervical tumor model system to determine the *in vitro* therapeutic index of new anti-cancer agents such as 17-AAG. Following 17-AAG treatment (150 nM), cells were exposed to ionizing radiation (IR) doses as indicated (2, 4, 6, or 8 Gy), trypsinized, and plated at various densities into compartments of a 6-well plate. After 10 days, colonies were stained and scored, and the surviving fraction was plotted versus IR dose. The solid stars represent cells treated with IR alone, whereas the solid triangles represent cells pretreated with 17-AAG for 12 hours. Curves are normalized to account for drug-induced cytotoxicity. The results presented represent the average of three separate experiments. Error bars around data points represent one standard deviation about the arithmetic mean, and statistical significance was established by Student's *t* test ($P < 0.05$).

experiments to determine if 17-AAG would enhance the tumor cell killing potential of IR in a human cervical tumor cell model system. This system was chosen for several specific and practical considerations. First, cervical tumors are a considerably more homogenous human malignancy, due to human papillomavirus (HPV) infection, than other tumor types. Second, the genetics of transformation clearly involves the HPV16-E6 and HPV16-E7 gene products, allowing for a detailed analysis of the interaction between anticancer agents and cervical cancer–specific oncogenes. In addition, using tissue culture cell lines that express HPV16-E6, HPV16-E7, or both, it is possible to make an *in vitro* determination as to whether the anticancer effects of 17-AAG are limited to transformed versus non-transformed cell lines. Finally, the cervix is easily accessible to repeated biopsies making tissue procurement and subsequent analysis of molecular markers possible.

In collaboration with James Mitchell, PhD (Radiation Biology Branch, CCR), and

Leonard Neckers, PhD (Urologic Oncology Branch, CCR), we initiated a series of cell biological, molecular, and biochemical studies to validate 17-AAG as a radiosensitizing agent in cervical tumors. Treatment of two human cervical carcinoma cell lines (HeLa and SiHa) with 17-AAG resulted in cytotoxicity, and when combined with IR, we observed a marked enhancement of radiation-induced cell killing (on the order of 70%, each effect with a temporal range of 6 to 48 hours after drug exposure). In addition, mouse xenograft *in vivo* models using 17-AAG at clinically achievable concentrations yielded results that paralleled the *in vitro* radiosensitization studies of both single and fractionated courses of irradiation. The increase in IR-induced cytotoxicity appears to be due to a combination of both programmed and non-programmed cell death, as determined by alterations in total levels of several pro-survival and apoptotic signaling proteins. Akt1, Erk-1, Glut-1, Her-2/neu, Lyn, protein kinase A, Raf-1, and vascular endothelial growth factor expression were downregulated in 17-AAG–treated cells, identifying these factors as molecular markers and potential therapeutic targets. Finally, a series of immortalized and HPV-transformed cell lines were used to demonstrate that the radiosensitizing effects of 17-AAG were limited to transformed cells, suggesting a possible differential cytotoxic effect (Figure 1). This work shows that altered HSP90 function induces significant tumor cytotoxicity and radiosensitization, suggesting a potential therapeutic utility.

Tumors exhibit considerable genetic plasticity and, as such, may quickly adapt to the cytotoxicity of such endogenous anticancer agents with finite molecular targets, suggesting the need for combining anticancer agents or using those with multiple targets of action. 17-AAG represents an agent that alters the intracellular function of multiple signaling factors shown to upregulate pro-survival pathways. As such, it is plausible that 17-AAG, through abrogation of pro-survival pathways and alteration of HSP90 function, may increase tumor cell death when combined with IR. This work identifies 17-AAG as a potential therapeutic agent that can be used at clinically relevant doses in conjunction with IR for the treatment of cervical tumors. In addition, pre-clinical *in vivo* data in mouse models validate its use. Preliminarily, we have identified several potential molecular therapeutic targets modulated by 17-AAG, as well as molecular markers that may be used in *in vivo* systems and in patient biopsies to verify its effects. Work to uncover additional signaling pathways, molecular markers, and therapeutic targets are under way.

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■ MOLECULAR BIOLOGY

The Dynamics of Gene Silencing

Cheutin T, Gorski SA, May KM, Singh PB, and Misteli T. *In vivo* dynamics of Swi6 in yeast: evidence for a stochastic model of heterochromatin. *Mol Cell Biol* 24: 3157–67, 2004.

At any given time, each cell in an organism only expresses a subset of its 25,000 or so genes. Distinct sets of genes that are active in one cell type may be permanently silenced in other types. Misregulation, leading to activity of a gene at the wrong time or in the wrong place, can be catastrophic and result in disease and cancer. The mechanisms involved in permanent silencing of genes are of greatest interest in our efforts to understand diseases and are likely to provide novel therapeutic strategies.

A critical factor in determining transcription activity appears to be the higher-order folding of the chromatin region at and near a gene. Active genes are usually found in open, decondensed chromatin regions, whereas silenced genes are frequently sequestered in highly condensed heterochromatin.

The mechanisms involved in permanent silencing of genes are of greatest interest in our efforts to understand diseases and are likely to provide novel therapeutic strategies.

It has generally been assumed that the closed nature of heterochromatin plays an important role in gene silencing by preventing the access of transcriptional activators to the genes located in heterochromatin. The molecular basis for heterochromatin is the methylation of lysine 9 of histone H3, one of the core histone chromatin proteins. A mechanism that has been evolutionarily conserved from the primitive unicellular yeast *Schizosaccharomyces pombe* to humans, this epigenetic modification serves as a binding site for structural heterochromatin proteins that maintain the highly condensed chromatin state. In mammals, the key structural heterochromatin protein is HP1, whereas in yeast it is swi6p.

Although much biochemical and molecular analysis has gone into understanding heterochromatin, we set out to probe the cell biological behavior of these genome regions to uncover how heterochromatin functions *in vivo*. We created a *S. pombe* strain in which the endogenous

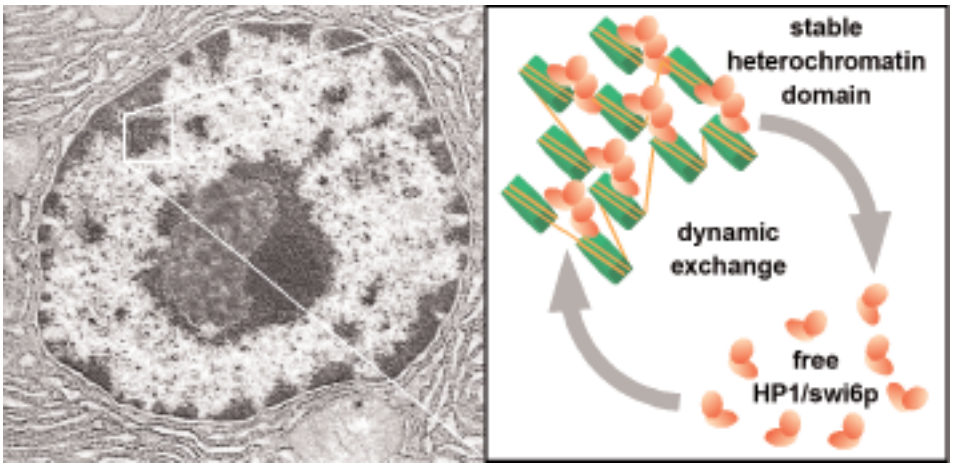

Figure 1. Heterochromatin (dark areas) is a major component of the eukaryotic cell nucleus. Evolutionarily conserved structural proteins (red) maintain the condensed state of heterochromatin by crosslinking neighboring nucleosomes. Despite their structural role, HP1 proteins (in mammalian cells) and swi6p (in *Schizosaccharomyces pombe*) are not statically bound to chromatin but are rapidly exchanged between chromatin and the nucleoplasmic space.

swi6p is replaced with a fully functional fluorescently tagged version. The fluorescent protein accumulated as expected in heterochromatin regions and allowed us to visualize and characterize for the first time the dynamics of these genome regions in living cells. When observed by time-lapse microscopy, we found that heterochromatin regions were stable, but were highly dynamic in that they easily traversed the nucleus in a matter of seconds. Thus, the yeast genome is highly dynamic *in vivo*.

It has generally been assumed that structural proteins of heterochromatin are rigidly bound to provide a static framework and in this way maintain the condensed chromatin state. To test this hypothesis, we measured the binding dynamics of swi6p in chromatin of living cells using *in vivo* photobleaching microscopy. Surprisingly, we found that swi6p only very transiently binds to heterochromatic regions and that each molecule is dynamically exchanged from chromatin every few seconds. By taking advantage of the power of yeast genetics, we were also able to determine that swi6p crosslinks nucleosomes by dimer-formation, whereby a histone-binding domain anchors swi6p molecules to chromatin and a protein-protein interaction domain crosslinks swi6p molecules bound to neighboring nucleosomes (Figure 1).

It is likely that the crosslinking ability of swi6p is essential for maintaining the condensed heterochromatin state.

These observations create the paradoxical situation that stable heterochromatin domains are maintained by highly dynamic structural components that generate a dynamic steady state (Figure 1). Maintenance of heterochromatin does not appear to involve a static structural framework, but rather the stochastic and rapid binding and unbinding of structural heterochromatin proteins. Since we had made similar observations in mammalian cells (Cheutin et al. *Science* 299, 721–5, 2003), our results suggest that this dynamic behavior of heterochromatin and its proteins is evolutionarily conserved and, thus, likely functionally relevant. We propose that the dynamic nature of heterochromatin organization is essential since it allows the rapid reactivation of silenced genes in response to altered environmental cues by simple competition between repressive heterochromatin proteins and transcriptional activators.

Uncovering the molecular and cell biological mechanisms involved in establishing and maintaining chromatin domains is critical to our understanding of gene expression. These mechanisms are also of utmost clinical relevance as indicated by the fact that inhibitors that interfere with histone acetylation, a different type of epigenetic modification of core histones, are currently being explored in clinical trials for treatment of several cancers including T-cell lymphoma and prostate cancer. The control of chromatin states is yet another example where insights into a basic cellular process are laying the foundation for innovative strategies in the treatment of cancers.

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A Role for Smad7 in Cancer Pathogenesis: Insight from a Mouse Cutaneous Carcinogenesis Model

Liu X, Lee J, Cooley M, Bhogte E, Hartley S, and Glick A. Smad7 but not Smad6 cooperates with oncogenic ras to cause malignant conversion in a mouse model for squamous cell carcinoma. *Cancer Res* 63: 7760–8, 2003.

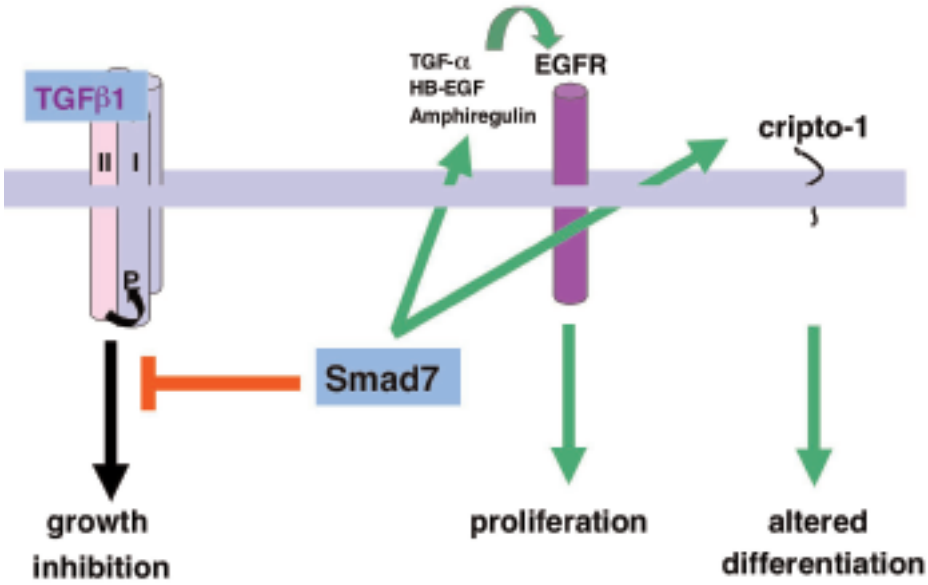

Figure 1. Smad7 targets multiple pathways that contribute to the malignant phenotype. TGFβ1, transforming growth factor β1; TGFα, transforming growth factor α; EGFR, epidermal growth factor receptor, HB-EGF, heparin-binding epidermal growth factor; P, phosphorylation of the type I receptor kinase by the type II receptor kinase.

How cells become insensitive to signals that negatively regulate cell growth is an important issue in cancer pathogenesis. Transforming growth factor β1 (TGFβ1) signaling is an important tumor suppressor pathway for early stages of many epithelial cancers, in part through its negative regulation of the cell cycle. Cancer cells have devised many different mechanisms for overcoming the growth regulation imposed by TGFβ1 signaling, including mutational inactivation of the TGFβ1 type II receptor and the intracellular effectors of the TGFβ1 signaling pathway, termed Smad proteins. Unlike most Smads, however, Smad7 is an inhibitor of TGFβ1 signaling that generates a physiologically relevant negative feedback loop in response to TGFβ1 and other growth factors. Smad7 blocks phosphorylation of signaling Smads such as Smad1, Smad2, and Smad3 by the activated type I receptor for a number of different TGFβ superfamily ligands including TGFβ1, activin, and bone morphogenic proteins (BMPs). Smad6, a closely related molecule, is a more specific inhibitor of BMP signaling.

When we began these studies, there were no direct experimental data linking Smad7 overexpression to increased risk of malignant conversion, although Smad7 is overexpressed in inflammatory bowel disease, chemically induced mouse epidermal papillomas and carcinomas, and human pancreatic and colon cancers. To test the hypothesis that Smad7 overexpression could accelerate premalignant progression, we generated retroviruses expressing mouse Smad7 and Smad6 and used them in a model of multistage cutaneous squamous cancer development. In this model, primary mouse keratinocytes are first transduced *in vitro* with a v-*ras*^{Ha} retrovirus to produce a genetic and biochemical model of the initiated epithelial cell. These cells can be grafted along with dermal fibroblasts onto the dorsal epidermis of a nude mouse where they will form a benign squamous papilloma. The effect of further *in vitro* genetic manipulations on tumor progression can then be determined *in vivo*.

When primary mouse keratinocytes were transduced with the Smad7 retrovirus in the absence of v-*ras*, cell proliferation was transiently enhanced *in vitro* and grafts of these cells formed hyperplastic skin. Surprisingly, these primary cells then began to express keratin 8 and 18, markers of simple epithelia, while expression of some normal squamous differentation markers, such as keratin 1 and keratin 10, was blocked. Importantly, aberrant expression of these simple epithelial keratins is a hallmark of malignant squamous cancers, suggesting that Smad7 alone was capable of driving some, but not all, aspects of neoplastic transformation.

In contrast, when Smad7 was overexpressed in the context of an activated *ras* oncogene, it caused *in vitro* immortalization of these cells as indicated by extended growth, reduced senescence, and increased frequency of calcium-resistant transformed colonies. These cells also exhibited the simple epithelial differentiation phenotype seen in the primary keratinocytes. When these Smad7/v-*ras*^{Ha} co-infected keratinocytes were grafted with dermal fibroblasts *in vivo*, they formed papillomas that rapidly progressed to squamous cell carcinomas, while pBabe/v-*ras*^{Ha} control grafts only formed benign papillomas. Surprisingly, grafted Smad6/v-*ras*^{Ha}—transduced keratinocytes also formed only benign papillomas, suggesting that inhibition of BMP signaling is not sufficient to drive v-*ras*—initiated keratinocytes to malignancy. However Smad7/v-*ras*^{Ha} tumors had defective nuclear localizaton of Smad2, Smad3, and Smad5, suggesting that the

signaling of other TGFβ superfamily ligands in addition to TGFβ1 was being blocked.

We wondered what downstream targets were altered in Smad7-overexpressing cells that could account for their altered rapid malignant conversion *in vivo*. We found that in Smad7-overexpressing keratinocytes, transforming growth factor α, heparin-binding epidermal growth factor, and amphiregulin, each of the epidermal growth factor (EGF)–like superfamily of growth factors, were induced and epidermal growth factor receptor tyrosine phosphorylation was also elevated, suggesting that these growth factors were providing an autocrine mitogenic stimulus. Additionally, cripto-1, of the EGF-cripto, FRL-1, and cryptic (EGF-CFC) family of signaling proteins, was induced by Smad7 in primary keratinocytes and in carcinomas derived from the Smad7/v-*ras*^{Ha}–transduced keratinocytes. Cripto-1 is a co-receptor for the TGFβ1 superfamily member, nodal, but by itself can block the signaling of activin, again a TGFβ superfamily ligand, although non-Smad–dependent pathways also exist.

We found that treatment of primary mouse keratinocytes with recombinant cripto-1 could induce keratin 8 and 18 expression, suggesting that expression of this growth factor is the primary mediator of the squamous-to-simple epithelial differentiation switch. Taken together, these results suggest three interconnected pathways by which Smad7 overexpression could accelerate tumor progression: inhibition of TGFβ superfamily signaling, induction of the mitogenic EGF-like superfamily of growth factors, and expression of a differentiation factor. These results showed for the first time that upregulation of Smad7 can profoundly alter the tumorigenic potential of primary epithelial cells. Given that this occurred with only a 2- to 3-fold increase in expression over endogenous levels, it is possible that small changes in expression of this inhibitory Smad could have a profound effect on the pathogenesis of human cancers.

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CANCER AND CELL BIOLOGY

Molecular Chaperones and Transcription Factor Mobility

Elbi C, Walker DA, Romero G, Sullivan WP, Toft DO, Hager GL, and DeFranco DB. Molecular chaperones function as steroid receptor nuclear mobility factors. *Proc Natl Acad Sci U S A* 101: 2876–81, 2004.

Nuclear receptors are hormone-dependent transcription factors that modulate gene expression in response to their cognate ligands. A central paradigm in endocrinology has been that ligand-activated receptors are statically bound to their regulatory sites in chromatin in the continued presence of hormone. We recently discovered that this concept is crucially flawed. When the interaction of the glucocorticoid receptor with hormone-response elements was examined by direct visualization in living cells, we found that the receptor only briefly resides on genes (McNally JG et al. *Science* 287: 1262–5, 2000). This concept was subsequently extended to coregulators that are recruited to promoters by the receptors (Becker M et al. *EMBO Rep* 3: 1188–94, 2002; Hager GL et al. *Curr Opin Genet Dev* 12: 137–41, 2002). These findings have led to a search for the mechanisms involved in the unexpected mobility of nuclear receptors; we have developed an *in situ* system to study this. In brief, cells containing green fluorescent protein (GFP)–tagged nuclear receptors are treated with digitonin to render the cell membrane permeable to macromolecules (Elbi C et al. *Sci STKE* 2004: 110, 2004; Elbi C et al. *Proc Natl Acad Sci U S A* 101: 2876–81, 2004). The nuclei remain relatively intact during this procedure, retain most of the nuclear receptor, and are transcriptionally competent. After permeabilization of cells, receptor mobility is lost (for both progesterone receptor and glucocorticoid receptor) but can be restored by the addition of a reticulocyte translation system. Molecular chaperones were quickly identified as the active factors in the reticulocyte lysate. As shown in Figure 1, panel A, permeabilization of the cell causes a complete loss of receptor mobility, as measured by the rate of fluorescence recovery in the bleach zone. The addition of a mixture of seven highly purified molecular chaperones leads to recovery of more than 80% of the mobility observed in living cells (Figure 1, panel B).

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In contrast, the equally mobile human centromere heterochromatin protein 1 alpha (HP1α) is refractory to addition of chaperones in permeabilized cells (unpublished results, Elbi and Hager). Furthermore, we have also shown that HP1α mobility is unaffected *in vivo* by inhibition of heat shock protein 90 function with geldanamycin, whereas the rapid exchange of glucocorticoid receptor is strongly inhibited by geldanamycin

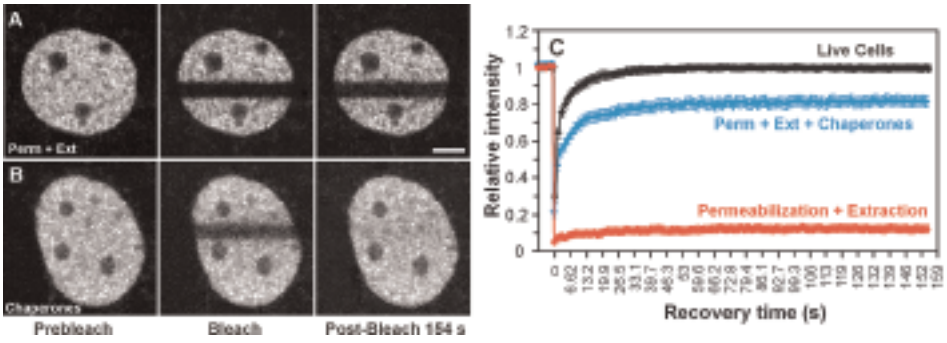

Figure 1. Restoration of glucocorticoid receptor (GR) nuclear mobility by molecular chaperones. Cells expressing green fluorescent protein (GFP)-tagged glucocorticoid receptor were treated with dexamethasone to induce nuclear translocation, then subjected to permeabilization and extraction (Elbi C et al. *Sci STKE* 2004: 110, 2004; Elbi C et al. *Proc Natl Acad Sci U S A* 101: 2876–81, 2004). Mobility of the nuclear receptor was determined by fluorescence recovery after photobleaching (FRAP) analysis, using a strip bleach across the diameter of the nucleus. *Panel A*: Permeabilized and extracted cells. *Panel B*: Permeabilized and extracted cells treated with a group of seven purified chaperones, including heat shock proteins 40, 70, and 90, as well as p23, FKBP51, CHIP, and p60/Hop proteins. *Panel C*: Quantitative FRAP analysis of GFP-GR in living cells, and in permeabilized and extracted cells—with and without the addition of chaperones. Perm, permeabilization; Ext, extraction. Scale bar, 3 μm.

in living cells. These findings introduce a new paradigm for chaperone involvement in nuclear protein mobility.

We currently entertain two general models for chaperone action. In the first model, termed the "chaperone cycle," many of the steroid/nuclear receptors exist in inactive chaperone complexes in the absence of ligand. Treatment of cells with hormone (i.e., the formation of active receptor) causes the disruption of these complexes. One tenet of this model is that these receptors cannot rebind ligands without reforming the chaperone complex. Thus, nuclear events involving ligand exchange (loss from the template, interaction with cofactor) could require a constant chaperone involvement to rebind hormone. Under this model, a chaperone role might be restricted to factors (nuclear receptors) that have this unusual requirement for ligand cycling.

In the second model, the role of chaperones is much more general. Although not frequently discussed, many nuclear receptors, including steroid receptors, typically have extensively unfolded regions. Induced folding of the glucocorticoid receptor AF1 domain has been shown to occur upon binding to a glucocorticoid-responsive element (Kumar R et al. *J Biol Chem* 276: 18146–52, 2001), and induced folding of the androgen receptor AF1 domain has been shown upon binding of a cofactor (Kumar R et al. *Biochemistry* 43: 3008–13, 2004). In principle, the extensive domain reorganizations that accompany these events could require participation of chaperone-refolding activities.

It now appears that multiple mechanisms are involved in the dynamic interaction of nuclear receptors with chromatin and other targets in the living cell. We recently reported that receptors are actively ejected from nucleoprotein templates during the process of chromatin remodeling (Nagaich AK et al. *Mol Cell* 14: 163–74, 2004), providing a third general mechanism for the mobility of these proteins, in addition to the two suggested chaperone-related mechanisms. The newly discovered mobility of nuclear receptors and their many coregulators with gene targets opens a new chapter in the biology of these important classes of regulatory proteins.

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■ ADMINISTRATIVE LINKS

NCI Combined Intramural Principal Investigator Retreat
The 2005 Retreat will be held on January 12 and 13, 2005, at the Bethesda North Hotel and Conference Center across from the White Flint Metro station. Registration questions should be directed to Jennifer Kwok (NCI-Bethesda, kwokj@mail.nih.gov), Julie Hartman (NCI-Frederick, jhartman@mail.ncifcrf.gov), or Barbara McElroy (SAIC-Frederick, mcelroy@mail.ncifcrf.gov).

New NIH Travel Policy Affects Large International Meetings
Planning to attend an international meeting this year? The NIH Office of Financial Management has issued new requirements for those meetings that appear on the Largely Attended Events List (http://camp.nci.nih.gov/admin/news/admin/200411/FY2005_LAE_listing.xls). Notifications of Foreign Travel (NFTs) for these meetings must be entered 35 days prior to the departure date. Travelers will not be permitted to attend the meeting if the NFT has not been entered by the 27th day prior to departure. For more details, visit the NCI Administrative Newsletter at http://camp.nci.nih.gov/admin/news/admin/200411/TravelUpdate.htm.

Cool Inhibitors for a Hot Target: Phosphatidylinositol Ether Lipid Analogues and the Serine/Threonine Kinase Akt

Castillo SS, Brognard J, Petukhov PA, Zhang C, Tsurutani J, Granville CA, Li M, Jung M, West KA, Gills JG, Kozikowski AP, and Dennis PA. Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues. *Cancer Res* 64: 2782–92, 2004.

Since its initial description in 1991, the serine/threonine kinase, Akt, has received increasing attention as a promising target for cancer therapy. Akt is the cellular homolog of the transforming viral oncogene, v-*Akt*, and regulates many essential cellular functions, including proliferation, migration, translation, and survival. Akt is a key player in cancer biology, considering that it is frequently detected in an active state in human cancers, is activated by carcinogens, and promotes resistance to chemotherapy or radiation. However, the study of Akt has thus far been limited by the lack of small-molecule inhibitors. To address this need, we have designed, synthesized, and characterized a series of compounds called phosphatidylinositol ether lipid analogues (PIAs) that were based on molecular modeling of the activation of Akt.

Akt becomes activated when the products of phosphatidylinositol 3-kinase (PI3K), $PI(3,4)P_2$ and $PI(3,4,5)P_3$, bind to the pleckstrin homology (PH) domain of Akt. This induces a conformational change in Akt and facilitates translocation to the plasma membrane. At the membrane, Akt becomes fully active after phosphorylation at two key residues. Although many efforts in industry and academia were already targeting the ATP domain to inhibit Akt, we felt we could contribute to the development of Akt inhibitors because PIAs target the PH domain of Akt and are structurally similar to $PI(3,4)P_2$ and $PI(3,4,5)P_3$.

PIAs are composed of three parts: a substituted inositol ring, a linker, and an ether lipid side chain. Our studies began with the synthesis of a series of PIAs that varied in each of these components. We performed cell-based screening for inhibition of Akt by 24 PIAs in breast and lung cancer cell lines whose Akt status was previously known. Five of the 24 PIAs rapidly inhibited Akt activation and the phosphorylation of multiple downstream substrates, including tuberin, 4EBP-1, p70 S6K, FKHR, AFX, c-Raf, and GSK-3β, without affecting kinases upstream of Akt. We determined that the biologic activity of PIAs depended on the nature of substitutions in the inositol ring, because a PIA composed only of the ether lipid backbone was inert. Computer modeling and docking studies predicted that PIAs bind to the PH domain of Akt with the inositol ring and the lipid side-chain in an altered position as compared with its endogenous substrates, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Figure 1). This suggested that PIAs could inhibit the conformational changes in Akt caused by binding of the products of PI3K, which might interfere with translocation of Akt. To confirm this, we showed that an active PIA inhibited insulin-like growth factor-I (IGF-I)– induced translocation of a green fluorescent protein (GFP)–tagged Akt construct from the cytoplasm to the

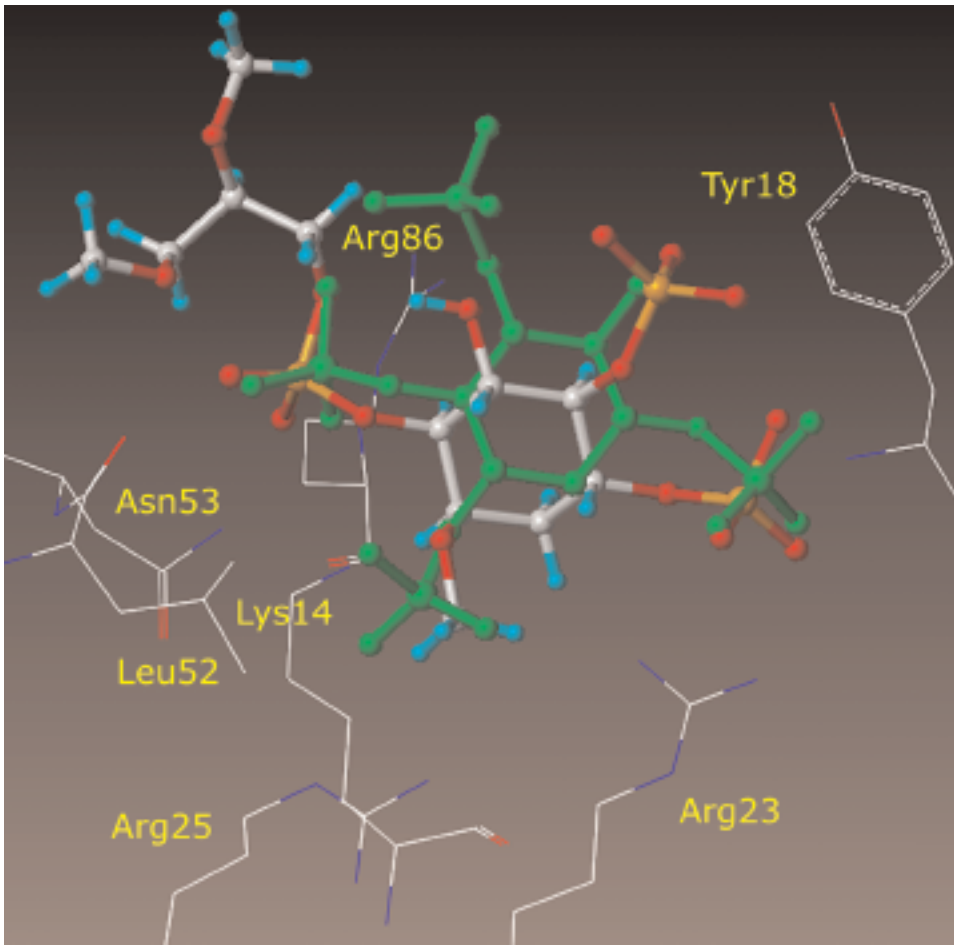

Figure 1. Overlay of 1,3,4,5-tetraphosphate, the head group of $PI(3,4,5)P_3$ (green ball and stick model), a product of phosphatidylinositol 3-kinase, and phosphatidylinositol ether lipid analogue 23 (PIA23) (other ball and stick model) in the pleckstrin homology (PH) binding site of Akt. PIAs were originally designed to interact with the PH domain of Akt and were later found to inhibit Akt activity, Akt translocation, and induce apoptosis in Akt-dependent cancer cells.

plasma membrane, indicating that inhibition of translocation is likely responsible for Akt inhibition.

We then administered PIAs to cancer cell lines that had varying dependence on Akt for survival. The five active PIAs increased apoptosis 20 to 30 fold in cell lines with high constitutive Akt activity, but only increased apoptosis 4 to 5 fold in cell lines with low levels of Akt activation. The induction of apoptosis was faster and greater than that observed in prior studies with these cell lines using a PI3K inhibitor or standard chemotherapeutic agents. Transfection of a constitutively active form of Akt that does not depend on binding of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ protected against PIA-induced apoptosis, showing that PIA-induced apoptosis depends on Akt inhibition. Together, these studies show that PIAs inhibit Akt and preferentially increase apoptosis in cancer cells with high levels of Akt activation.

What lessons can be learned from these studies? First, molecular modeling can be a valuable tool to guide rational synthesis of new molecules. Our "hit rate" of 25% is far higher than that typically achieved by screening large compound libraries. Second, kinases can be inhibited through multiple mechanisms that involve different regions of the protein. Future studies will determine whether targeting the PH domain truly offers greater specificity than that offered by targeting the kinase domain. Perhaps the most critical question is whether PIAs will become effective therapeutic agents for cancers that have Akt activation. Many issues must be addressed before this milestone is reached. Because many normal tissues and processes also rely on Akt, the therapeutic index for any approach targeting Akt may be small. However, conditions uniquely encountered by solid tumors such as low oxygen tension or low pH are known to promote Akt activation, which could indicate that tumor cells have increased reliance on Akt activation for survival. The demonstration of *in vivo* efficacy, determination of sensitive tumor types, and evaluation of any potential secondary targets will determine whether the promise of inhibiting Akt with PIAs can be fulfilled.

...we have designed, synthesized, and characterized a series of compounds called phosphatidylinositol ether lipid analogues (PIAs) that were based on molecular modeling of the activation of Akt.

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